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In vivo tumor-targeted dual-modal fluorescence/CT imaging using a nanoprobe co-loaded with an aggregation-induced emission dye and gold nanoparticles

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ABSTRACT

As an intensely studied computed tomography (CT) contrast agent, gold nanoparticle has been suggested to be combined with fluorescence imaging modality to offset the low sensitivity of CT. However, the strong quenching of gold nanoparticle on fluorescent dyes requires complicated design and shielding to overcome. Herein, we report a unique nanoprobe (M-NPAPF-Au) co-loading an aggregation-induced emission (AIE) red dye and gold nanoparticles into DSPE-PEG_{2000} micelles for dual-modal fluorescence/CT imaging. The nanoprobe was prepared based on a facile method of "one-pot ultrasonic emulsification". Surprisingly, in the micelles system, fluorescence dye (NPAPF) efficiently overcame the strong fluorescence quenching of shielding-free gold nanoparticles and retained the crucial AIE feature. *In vivo* studies demonstrated the nanoprobe had superior tumor-targeting ability, excellent fluorescence and CT imaging effects. The totality of present studies clearly indicates the significant potential application of M-NPAPF-Au as a dual-modal non-invasive fluorescence/X-ray CT nanoprobe for *in vivo* tumor-targeted imaging and diagnosis.

1. Introduction

The past decade has witnessed the rapid development of imaging nanoprobes, which are able to provide physiological and pathological information with high sensitivity and specificity for disease diagnosis [1–5]. However, single imaging techniques are only able to supply limited information, which is sometimes insufficient for accurate imaging diagnosis [6–9]. Due to this drawback, dual-modal nanoprobes, which combine the advantages of each imaging modality, have attracted great attention in recent years [10–12].

As a clinically approved imaging modality, X-ray computed tomography (CT) possesses the incomparable advantages of high spatial resolution and unlimited penetration depth [13,14]. Among various CT contrast agents, gold nanoparticle owns an extremely high X-ray absorption coefficient, regardless its preparation method, shape, diameter, etc. [15]. However, CT imaging modality shows an inherent disadvantage of low sensitivity [13]. Hence, in order to develop a complementary dual-modal imaging probe, some imaging modalities with high sensitivity need to be combined with CT.

Among all other imaging modalities, red to near-infrared (Red-NIR, 600–900 nm) fluorescence imaging is highly attractive for early

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non-invasive detection of cancers because it has lots of excellent advantages such as high sensitivity, low consumption and facile operation [16,17]. Fluorescence probes with long wavelength emission are highly desirable for detection, because biological tissues show relatively low absorption and autofluorescence in this region [18,19]. Therefore, it is advisable to fabricate sensitive fluorescence nanoprobes using Red-NIR dyes. However, fluorescence imaging suffers from its own shortcomings, including low spatial resolution and limited penetration depth even at Red-NIR wavelengths [7].

Based on the analysis above, it would be ideal to combine CT and fluorescence imaging together to develop a complementary imaging method with high spatial resolution and high sensitivity. However, conventional fluorescence dyes always suffer from low brightness due to a notorious phenomenon known as aggregation caused quenching (ACQ) [20]. In addition, gold nanoparticles (Au NPs), an intensely explored CT contrast agent, are well known as a strong quencher of fluorescence dyes [21]. Thus, the fabrication of fluorescence/CT dual-modal nanoprobes has been a great challenge [22,23]. In 2001, Tang et al. unveiled a fluorescent molecule which emitted strong fluorescence when aggregated and thus presented an early example of aggregation-induced emission (AIE) [16]. These unique AIE fluorescent molecules are resistant to self-quenching and provide a promising solution for fabricating new imaging probes with superior performance both *in vitro* and *in vivo*. Furthermore, if the size of a nanoprobe is appropriate, it is ideal for tumor-targeted imaging because of the enhanced permeation and retention (EPR) effect [24]. To date, however, there is rare report in the literature of whether the AIE dyes could overcome the quenching effect of gold nanoparticles to fabricate highly efficient dual-modal fluorescence/CT nanoprobes.

Herein, we fabricated a unique nanoprobe by co-loading the AIE red dye (NPAPF) and gold nanoparticles into the well-known FDA-approved material 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) micelles. The nanoprobe was prepared by a facile method of "one-pot ultrasonic emulsification". Unexpectedly, the utilized AIE dye (NPAPF) showed relatively enhanced emission in the as-prepared micelles system despite the existence of gold nanoparticles, which guaranteed its efficient fluorescence imaging effect. *In vitro* and *in vivo* results demonstrate the nanoprobe has good biocompatibility, long blood circulation half-life, superior tumor-targeting ability, and excellent fluorescence and CT imaging effects. To our knowledge, this is the rarely reported fluorescence/CT dual-modal micelles system in which red fluorescence dye (NPAPF) reserves its AIE feature in the presence of shielding-free gold nanoparticles.

2. Materials and methods

2.1. Materials and instruments

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) was purchased from Avanti Polar Lipids (Alabaster, AL). Bis(4-(N-(2-naphthyl) phenylamino) phenyl)-fumaronitrile (NPAPF) was synthesized according to a previous report [25]. 4-bromophenylacetonitrile, N-phenyl-substituted amine, $Pd(OAc)_2$, Cs_2CO_3 and P(*t*-Bu)$_3$ were purchased from J&K Scientific Ltd. Diethyl ether, sodium, dichloromethane, petroleum, methanol, toluene, chloroform, metal-oxide-semiconductor (MOS) grade nitric acid, hydrochloric acid and hydrogen peroxide were purchased from the Beijing Chemical Reagents Institute (Beijing, China). Au NPs were prepared according to the literature [26]. Gold chloride trihydrate (99.9%, $HAuCl_4 \cdot 3H_2O$) and oleylamine (70%) were purchased from Energy-Chemical (Beijing, China). Au standard solution (1000 μg/mL) was obtained from the National Analysis Center for Iron and Steel (Beijing, China). All of the glasswares used for the preparation and storage of Au NPs were pre-cleaned with aqua regia ($HCl:HNO_3$ = 3:1, v/v). All of the chemicals were used without further purification, and Milli-Q water (18.2 MΩ) was used throughout this study.

The micelles were prepared on a KQ-100DE ultrasonic cleaner (Kunshan, China). Particle size was determined by dynamic light scattering (DLS) using a Malvern Zeta sizer ZS90 instrument (Worcestershire, U.K.). TEM images of all micelles were obtained through a FEI Tecnai G2 F20 S-Twin TEM (Hillsboro, OR). Emission spectra were characterized by a Fluoromax4 spectrometer (Horiba Jobin Yvon, Edison, NJ). UV–Vis–NIR spectra were obtained by a LAMBDA 950 UV/Vis/NIR spectrometer (PerkinElmer, U.S.A.). Cellular uptake was characterized with a Zeiss LSM510 confocal laser microscope (Carl Zeiss Shanghai Co. Ltd, Shanghai, China) and an Attune® acoustic focusing cytometer (Applied Biosystems, Life Technologies, Carlsbad, CA). The biodistribution of Au in tumor tissues and organs was determined by NexION 300× inductively coupled plasma mass spectrometry (ICP-MS), (PerkinElmer, U.S.A.). *In vitro* and *in vivo* fluorescence images were collected by a Maestro 2 multi-spectral imaging system (Cambridge Research & Instrumentation, U.S.A.). *In vitro* and *in vivo* CT images were obtained by SPECT/CT scanning system (Triumph X-SPECT/X-O CT, GMI Company, U.S.A.).

2.2. Preparation and characterization of M-NPAPF-Au

Micelles loaded with NPAPF, or Au NPs, or NPAPF and Au NPs together (shortened as M-NPAPF, M-Au, M-NPAPF-Au, respectively) were prepared by "one-pot ultrasonic emulsification". Briefly, DSPE-PEG_{2000} (8 mg) powder was placed into a round-bottom flask, and then Au NPs in chloroform (2 mg/L, 0.5 mL) and NPAPF in chloroform (1 mg/mL, 1 mL) were added into the flask and mixed thoroughly until the DSPE-PEG_{2000} was completely dissolved. After that, 10 mL Milli-Q water (18.2 MΩ) was added into the mixture, then the flask was placed in an ultrasonic bath cleaner for about 10 min ultrasonic emulsification at 100 W power. The organic solvent was then removed by evaporation while stirring in a fume hood overnight at room temperature. Following similar procedures, M-NPAPF was prepared without using Au NPs and M-Au was prepared without using NPAPF.

The morphology of M-NPAPF, M-Au and M-NPAPF-Au was examined using a Tecnai G2 20 STWIN transmission electron microscope with a 200 kV acceleration voltage. The distribution of hydrodynamic particle size was measured by a Malvern Zeta sizer ZS90.

2.3. Analysis of AIE properties and quantum yield determination

The absorption spectra of M-NPAPF, M-Au and M-NPAPF-Au were determined using a UV/Vis spectrometer and the photoluminescence (PL) spectrum was measured with a luminescence spectrometer. The concentration of CH_3CN in the CH_3CN/H_2O mixture ranged from 0% to 99.9%. Fluorescence quantum yields (QY) were determined according to the published reports in the literature [27]. Rhodamine B was chosen as a standard molecule. All UV–Vis absorption values were measured at a wavelength of 500 nm, and all PL spectra data were obtained with an excitation wavelength of 500 nm. QY values were calculated according to the following equation [28]:

$$\varphi_u = \varphi_s{}^*(F_u/F_s)^*(A_s/A_u) \quad (1)$$

(φ_u: quantum yield of test substance; φ_s: quantum yield of standard substance; F_u: integrated fluorescence intensity of test substance; F_s: integrated fluorescence intensity of standard substance; A_u: UV absorption of test substance; A_s: UV absorption of standard substance.)

2.4. Cytotoxicity studies

The cytotoxicity of M-NPAPF-Au was evaluated by MTT assay. BALB/c mice colon adenocarcinoma cells (CT26) were incubated in RPMI 1640 medium, human hepatocellular carcinoma cells (HepG2) and normal human liver cells (L02) were incubated in DMEM medium, which were all supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. The cells were seeded at 5×10^3 per well into a 96-well plate overnight, respectively, then incubated with 100 μL medium containing various concentrations of M-NPAPF-Au ranging from 37.5 to 600 μg/mL for 24 h. After that, the medium was replaced with medium containing MTT (0.5 mg/mL, 100 μL) for 4 h, and then the MTT medium was replaced with 100 μL DMSO. The absorbance was measured at 570 nm with a reference wavelength of 630 nm using an Infinite M200 microplate reader (Tecan, Durham, U.S.A.). Untreated cells were used as control. All experiments were carried out with five replicates.

2.5. Flow cytometric analysis

CT26 cells were seeded at 1×10^5 cells per well into 6-well plates, then incubated with 2 mL M-NPAPF-Au (200 μg/mL). Cells were harvested at 6, 12, 24 h, and then analyzed using an Attune® acoustic focusing cytometer.

2.6. Confocal laser scanning microscopy (CLSM) imaging

CT26 cells were seeded with a density of 5×10^4 per dish in 35 mm glass microscopy dishes and incubated overnight at 37 °C. After washing with PBS, cells were incubated with 1 mL M-Au, M-NPAPF or M-NPAPF-Au (in each case the concentration of micelles was 200 μg/mL) for 24 h. Excess micelles were removed by washing three times with PBS, then cells were stained with 200 μL DAPI (20 μg/mL) for 1 min, washed three times with PBS (100 μL) and observed by confocal laser scanning microscopy (CLSM) with laser excitation at 488 nm. Fluorescence was collected at wavelengths from 660 nm to 750 nm.